CONSTRUCTION OF MAMMALIAN EXPRESSION VECTOR CONTAINING SPECIFIC TP53 MUTATION BY SITE DIRECTED MUTATION

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

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TO WHOM IT MAY CONCERN

This is to certify that **Ms. Debolina Bose**, a student of the Department of Biotechnology, Sister Nivedita University, has successfully completed her long-term training at **ATGC Diagnostics Pvt. Ltd., West Bengal, India**, in the domain of Molecular Biology, from **4th January 2025 to 16th May 2025.**

During this period, she was engaged in a research project titled **"Construction of mammalian expression vector containing specific TP53 mutation by site directed mutagenesis"** under the supervision of **Dr. Susanta Roychoudhury**, Principal Investigator (PI) of the project. The project involved the design and development of a mammalian expression system harbouring a specific TP53 gene mutation using site-directed mutagenesis. Ms. Bose was trained in and performed various core molecular biology techniques including restriction digestion, ligation, plasmid isolation, bacterial transformation, recombinant plasmid construction, and agarose gel electrophoresis. She also gained exposure to the principles of site-directed mutagenesis, primer design, vector construction.

Ms. Bose demonstrated a high level of enthusiasm, responsibility, and dedication throughout the training. Her ability to grasp new techniques quickly and apply them effectively in the laboratory was commendable. She maintained professionalism and contributed positively to the lab environment, both individually and as a team member.

We appreciate her efforts and wish her continued success in her academic and professional pursuits.

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "**Construction of mammalian expression vector containing specific tp53 mutation by site directed mutagenesis**" in partial fulfilment of the requirements for the award of the degree of **Master of Science** in **Biotechnology** is an authentic record of my own work carried out during the period from **4th January, 2025** to **16th May, 2025** under the supervision of **Dr. Susanta Roychoudhury**, **Director of ATGC Diagnostics Pvt. Ltd.**

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Debolina Bose
06.06.2025

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TO WHOM IT MAY CONCERN

This is to certify that the project report titled ""**Construction of Mammalian Expression Vector containing specific tp53 Mutation by Site Directed Mutagenesis"** submitted by Ms. Debolina Bose (Registration No.:-230020260472, Enrollment No.:- 2322210002026) to the Department of Microbiology/Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by him/her for the partial fulfillment of the requirements for the Master of Science degree.

Date: 03.06.2025

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ABSTRACT

The TP53 gene is one of the most studied tumour suppressors due to its vital role in controlling cell growth and preserving genomic integrity. Among the many mutations reported in cancers, the R248Q substitution is notably recurrent and functionally significant. This project focused on introducing the R248Q mutation into a mammalian expression vector containing the wild-type TP53 gene. The mutation was successfully incorporated and validated, resulting in a plasmid construct that mimics a common cancer-associated alteration. The study highlights the practical application of molecular techniques to modify specific genetic sequences with precision. While the experimental process involved careful planning and verification, the outcome reflects a straightforward and effective strategy to model known mutations at the molecular level. The generated construct offers a representation of how a single nucleotide change can impact a gene known for its protective role in human cells. This work adds to the understanding of TP53 mutation modelling and reflects the importance of detailed molecular research in uncovering the complexities of gene function and alteration.

KEYWORDS

- TP53 Mutation
- Site-Directed Mutagenesis
- R248Q Mutation
- Mammalian Expression Vector
- p53 Protein
- Plasmid Construction
- Cancer Genetics

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1. INTRODUCTION

Site-directed mutagenesis (SDM) is a powerful molecular biology technique that enables the precise alteration of specific DNA sequences at predetermined locations. This method allows researchers to introduce targeted mutations such as point mutations, insertions, or deletions into a gene of interest, facilitating the study of gene function, protein structure, and interactions. SDM has become an indispensable tool in genetic engineering, protein engineering, and functional genomics, providing insights into the molecular mechanisms underlying various biological processes and diseases.

Located on chromosome 17p13.1, the TP53 gene encodes the p53 protein, a vital tumor suppressor that plays a key role in preserving genomic stability. It manages the cell's response to DNA damage by controlling processes such as cell cycle arrest, DNA repair, programmed cell death, and cellular aging. Due to its pivotal function in controlling cell proliferation and preventing malignant transformation, TP53 is often referred to as the "guardian of the genome." Alterations in the TP53 gene are among the most prevalent genetic abnormalities in human cancers, detected in more than half of all tumor types. These mutations frequently lead to loss of normal p53 function, allowing cells with damaged DNA to evade growth arrest and apoptosis, thereby promoting tumorigenesis.

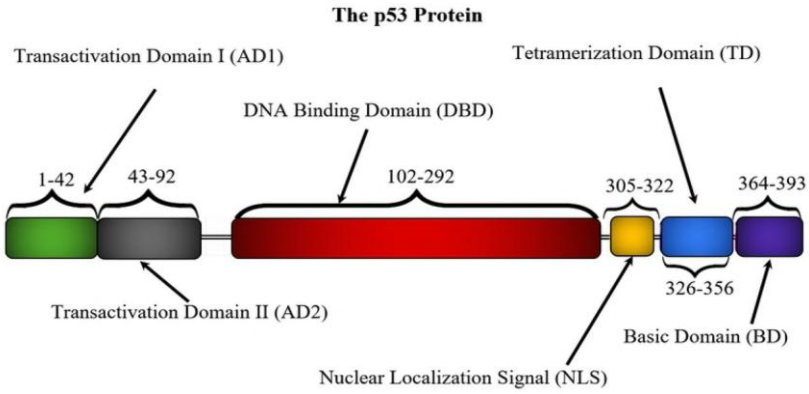


Figure 1.1:- Various Domains of p53 protein

Among the numerous mutations identified in TP53, the R248Q mutation is a prominent hotspot mutation located within the DNA-binding domain of the p53 protein. This mutation results from a single nucleotide substitution, changing the codon CGG (arginine) to CAG (glutamine) at position 248. Arginine at position 248 plays a critical role in DNA binding, and its substitution severely disrupts p53's capacity to attach to DNA and initiate transcription of its target genes. The R248Q mutation is associated with dominant-negative effects over wild-type p53 and gain-of-function oncogenic properties, contributing to cancer progression,

metastasis, and resistance to chemotherapy.

Given the biological and clinical significance of the R248Q mutation, constructing a mammalian expression vector harboring this specific mutation is essential for functional studies. Such a vector allows for the expression of mutant p53 in mammalian cells, enabling detailed investigation of its effects on cellular pathways, tumorigenic potential, and response to therapeutic agents. Site-directed mutagenesis provides a reliable and efficient approach to introduce the R248Q mutation into the TP53 gene within a plasmid vector, facilitating downstream functional assays.

This study aims to construct a mammalian expression vector containing the TP53 R248Q mutation by employing PCR-based site-directed mutagenesis. The parental wild-type TP53 plasmid will be amplified in Escherichia coli DH5α cells, followed by mutagenesis PCR using specifically designed primers to introduce the desired codon change. Confirmation of successful mutagenesis will be performed through restriction enzyme digestion and DNA sequencing. The resulting mutant vector will serve as a valuable tool for further research into the molecular mechanisms of mutant p53-driven cancers and the development of targeted therapies.

2. REVIEW OF LITRATURE

Advances in molecular cloning and gene editing have made it possible to introduce specific mutations into mammalian genes, providing powerful tools for studying the molecular basis of diseases such as cancer. One of the most impactful applications of these technologies is the construction of mammalian expression vectors carrying clinically relevant mutations, such as those found in the TP53 tumor suppressor gene. This project focuses on the TP53 gene, which encodes the p53 protein—a central player in tumor suppression—and utilizes site-directed mutagenesis to introduce a clinically significant mutation.

TP53, located on chromosome 17p13.1, is widely regarded as the "guardian of the genome." The p53 protein it encodes acts as a transcription factor that regulates genes involved in cell cycle arrest, apoptosis, DNA repair, and senescence. Under normal conditions, p53 remains at low levels due to rapid degradation, but upon cellular stress or DNA damage, it accumulates in the nucleus and initiates protective responses. This critical function is underscored by the fact that TP53 is the most frequently mutated gene in human cancers, with mutations observed in over half of all tumors (Olivier et al., 2010).

The p53 protein consists of several domains: an N-terminal transactivation domain, a central DNA-binding domain, and a C-terminal tetramerization domain. The DNA-binding domain is particularly important, as it allows p53 to recognize and bind to specific DNA sequences, thereby controlling the expression of downstream genes. Most cancer-associated mutations, including the R248Q mutation studied here, occur within this domain and disrupt p53's ability to bind DNA and regulate gene expression.

Missense mutations in TP53 are the most common type found in cancers. These mutations often result in the production of full-length but dysfunctional proteins. Some mutant forms, such as R248Q, not only lose their tumor-suppressive function but also gain new oncogenic properties—promoting cell survival, proliferation, and resistance to therapy (Brosh & Rotter, 2009). The R248Q mutation specifically changes an arginine to a glutamine at position 248, a residue that directly contacts DNA. This alteration severely impairs p53's DNA-binding ability and is associated with aggressive tumor phenotypes (Cho et al., 1994).

Site-directed mutagenesis (SDM) is a cornerstone technique in molecular biology that enables targeted changes to DNA sequences. By designing primers that introduce specific nucleotide substitutions, researchers can recreate disease-associated mutations or study protein structure-function relationships. The PCR-based SDM method is widely used for its

efficiency and precision. After amplification, DpnI digestion is employed to remove parental, methylated DNA, enriching for the mutated plasmid (Zheng et al., 2004). SDM has been instrumental in generating mutant constructs for functional studies, drug screening, and therapeutic development.

The construction of a mammalian expression vector carrying a specific TP53 mutation involves several key steps. First, the wild-type TP53 gene is cloned into a suitable plasmid backbone. In this project, mutagenic primers were designed to introduce the R248Q mutation (CGG to CAG) during PCR amplification. The amplified product is treated with DpnI to digest the original template, and the mutated plasmid is then transformed into competent E. coli cells for propagation.

Verification of the construct is essential. Plasmid DNA is isolated from bacterial colonies and analyzed by restriction digestion—using enzymes such as EcoRI—to confirm the presence and integrity of the insert. Sequencing is performed to ensure the correct mutation has been introduced and that no unintended changes have occurred. These validation steps are critical before proceeding to functional assays in mammalian cells.

Mammalian expression vectors play a crucial role in modern biomedical research by enabling scientists to express specific genes in a controlled and physiologically relevant environment. Unlike bacterial systems, mammalian cells provide the necessary post-translational modifications, folding mechanisms, and cellular context required for human proteins to function accurately. This is particularly important when studying complex regulatory proteins like p53, whose activity depends heavily on its interactions with other cellular components and its regulation through phosphorylation, acetylation, and other modifications. By using mammalian expression vectors, researchers can more faithfully replicate the conditions found in human tissues, allowing for more accurate insights into gene function, disease mechanisms, and potential therapeutic responses. These vectors not only facilitate the study of mutant proteins like p53-R248Q but also serve as platforms for testing drug candidates, developing gene therapies, and tailoring treatments to individual genetic profiles—bringing us closer to truly personalized medicine.

Mutant p53 constructs, such as those harboring the R248Q mutation, are invaluable for dissecting the molecular mechanisms of tumorigenesis. By expressing these constructs in mammalian cells, researchers can study the effects of specific mutations on cell cycle regulation, apoptosis, and response to DNA damage. These models also provide platforms for

screening drugs that restore wild-type p53 function or selectively target cells with mutant p53.

Furthermore, understanding the behavior of mutant p53 proteins has direct clinical implications. For example, certain p53 mutations are associated with resistance to chemotherapy, influencing treatment strategies and patient outcomes. The knowledge gained from these studies supports the development of personalized medicine approaches in oncology.

3. MATERIALS

1. pCMV Neo Bam p53 wt plasmid (https://www.addgene.org/16434/)
2. E. coli DH5α competent cells
3. LB (SRL)
4. Agar agar (SRL)
5. Ampicillin (100mg/ml)
6. EcoRI restriction enzyme (Thermo)
7. EcoRI 10X Buffer (Thermo)
8. Pfu DNA Polymerase (Agilent)
9. 10X Buffer (Agilent)
10. DpnI restriction enzyme (Agilent)
11. DpnI 10X Buffer (Agilent)
12. PCR purification kit (SLS)
13. Plasmid Mini Prep kit (SLS)
14. Custom primers (SDM) & (SEQUENCING)
15. Taq Polymerase (TaKaRa)
16. 10X Buffer (TaKaRa)
17. DNTP Mix (TaKaRa)
18. Agarose Powder
19. Ethidium Bromide
20. 1X TAE Buffer
21. Transformation Buffer (SRL)

4. METHADOLOGY

4.1. PLASMID ISOLATION

Amplifying the parental plasmid DNA (pCMV Neo Bam p53 wt) to obtain sufficient quantities for downstream applications such as SITE DIRECTED MUTAGENESIS, SEQUENCING, etc.

4.1.1. COMPETENT CELL PREPARATION

1. Primary Culture:

- Inoculate a single DH5α colony into 5 mL LB broth.
- Incubate overnight at 37°C (130 rpm).

2. Secondary Culture:

- Transfer 500 µL of primary culture into 5 mL fresh LB broth.
- Incubate at 37°C for ~1.5 hours (235 rpm) until OD600 reaches 0.4–0.6.

3. Competent Cell Preparation:

- Centrifuge 1.5 mL of the bacterial culture (in duplicate) at 10,000 rpm for 1 minute to pellet the cells.
- Resuspend in 500 µL cold transformation buffer. Chill on ice 15 min.
- Pellet again; resuspend in 200 µL cold transformation buffer.

4.1.2. TRANSFORMATION

- Add 2 µL of the pCMV Neo Bam p53 wild-type plasmid to the competent cells and mix gently...
- Ice 10 min → heat shock at 42°C for 90 sec → ice 5 min.
- Add 800 µL LB; recover at 37°C, 45 min (shaking).
- Plate 200 µL on LB agar + ampicillin.
- Incubate overnight at 37°C.

4.1.3. PLASMID ISOLATION

1 Firstly, suspend the bacterial pellet in 250 µL of Buffer P1 that contains RNase A. 2. Add 250 µL Buffer P2, mix gently by inversion.

3. Add 350 µL Buffer PN3, mix thoroughly.

4. Centrifuge at ≥10,000 rpm for 10 min.

5. Transfer supernatant to spin column.

6. Centrifuge the given sample at 10,000 rpm or more for 1 minute straight, then discard the liquid flow-through.

7. Add 0.5 mL of Buffer PW1 to wash the column then spin the column again, and gently remove the flow-through.

8. Wash with 0.7 mL Buffer PW2, centrifuge.

9. Repeat centrifugation to remove residual wash buffer.

10. Elute DNA with 50 µL nuclease free water; centrifuge and collect.

4.1.4. AGAROSE GEL ELECTROPHORESIS

A 1% agarose gel was prepared by dissolving 0.3 g of agarose in 30 mL of 1X TAE buffer. After cooling the molten agarose to approximately 60°C, Ethidium Bromide was added to a final concentration of 1X after partially cooling it down. The gel was cast in a tray with a comb and allowed to solidify.

The isolated plasmid sample (6 µL) was mixed with 1 µL of 6X loading dye and loaded into the well alongside a 1 kb DNA ladder. Electrophoresis was performed in 1X TAE buffer at 80 V for 60 minutes. The gel was then visualized under a UV transilluminator, and the image was captured using a gel documentation system.

Table 4.1 :- Concentrations of pCMV- Neo-Bam p53 wt plasmid

Sl.No.	**pCMV Neo Bam p53 wt**	**Concentration**
1.	Isolated plasmid (1)	2.14 µg/µL
2.	Isolated plasmid (2)	2.30 µg/µL

4.2. RESTRICTION DIGESTION OF PLASMID

To confirm the identity of the amplified pCMV-Neo-Bam p53 wt plasmid by restriction digestion using the EcoRI enzyme, which cuts at three known sites and yields three distinct DNA fragments.

Table 4.2: Restriction Digestion Reaction Setup

COMPONENTS	VOLUME
PLASMID DNA(2.30 µg/µL)	2 µL
EcoRI ENZYME	1 µL
10X BUFFER	2 µL
NUCLEASE- FREE WATER	15 µL
TOTAL	20 µL

The reaction mixture was incubated at 37°C for 2 hours in a water bath.

4.2.1. AGAROSE GEL ELECTROPHORESIS

A 1% agarose gel was prepared by dissolving 0.3 g of agarose in 30 mL of 1X TAE buffer. After cooling the molten agarose to approximately 60°C, Ethidium Bromide was added to a final concentration of 1X after partially cooling it down. The gel was cast in a tray with a comb and allowed to solidify.

The digested plasmid sample (10 µL) was mixed with 2 µL of 6X loading dye and loaded into the well alongside a 1 kb DNA ladder. Electrophoresis was performed in 1X TAE buffer at 80 V for 60 minutes. The gel was then visualized under a UV transilluminator, and the image was captured using a gel documentation system.

Expected Bands: ~3.6 kb, ~2.8 kb, and ~1.8 kb

4.3. SITE-DIRECTED MUTAGENESIS PRIMER DESIGNING

The objective of this step was to design mutagenic primers for the introduction of a specific point mutation—R248Q (CGG → CAG)—in the TP53 gene using a PCR-based site-directed mutagenesis strategy. At codon 248 of the TP53 protein, this mutation causes arginine (R) to be substituted by glutamine (Q).

Step 1: Identification of the Mutation Site

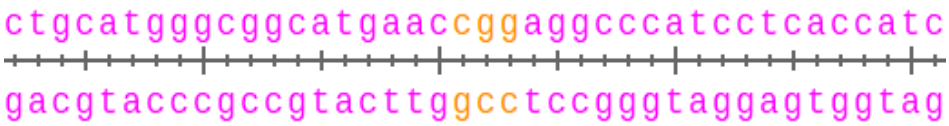


Figure 4.1:- Mutation site in the p53 sequence

The codon for arginine at position 248 (CGG) was identified within the TP53 sequence. The corresponding nucleotide sequence in the plasmid was located using SnapGene.

Step 2: Determination of Desired Codon Change

At codon 248 of the TP53 protein, this mutation causes arginine (R) to be substituted by glutamine (Q).

Step 3: Extraction of Flanking Sequences

Exact 19 nucleotides upstream and downstream of the target nucleotide were extracted to ensure appropriate primer binding during PCR amplification.

Step 4: Primer Design

The mutation was centrally located within the forward primer, and the reverse primer was the exact reverse complement. Both primers were between 39 bp in length, with GC content 63% , and a Tm 72.3°C, suitable for high-fidelity PCR.

Table 4.3:- Information about Forward and Reverse Primer.

FORWARD PRIMER	REVERSE PRIMER
5′-CTGCATGGGCGGCATGAAC**CAG**AGGCCCATCCTCACCATC-3′	5′-GATGGTGAGGATGGGCCT**CTG**GTTCATGCCGCCCATGCAG-3′
GC CONTENT :- 63%	GC CONTENT :- 63%
Tm 72.3°C	Tm 72.3°C
LENGTH:- 39 bp	LENGTH:- 39 bp

4.4. MUTAGENESIS PCR

The PCR was carried out using Pfu DNA polymerase, known for its proofreading activity and high-fidelity amplification, to minimize errors during synthesis. The reaction was set up in a final volume of 25 µL. Each reaction mixture contained 50 ng of plasmid DNA (wild-type TP53), 1 µL each of forward and reverse primers (125ng), 2 µL of dNTP mix (25 mM each), 2.5 µL of 10X high-fidelity buffer, and 0.5 µL of Pfu DNA polymerase. The remaining volume was adjusted with nuclease-free water.

Table 4.4:- Stock and final concentrations of three components

S.No.	COMPONENTS	STOCK CONCENTRATION	FINAL CONCENTRATION
1.	Forward Primer	1.22 µg/µL	1.25 µg/µL
2.	Reverse Primer	1.39 µg/µL	1.25 µg/µL
3.	Template DNA	2.30 µg/µL	1.00 µg/µL

Table 4.5:-Components and their volume for Site Directed Mutagenesis PCR.

COMPONENTS	VOLUME
NUCLEASE FREE WATER	17.5 µL
AGILENT 10X BUFFER	2.5 µL
DNTP (2.5Mm Each)	2 µL
FORWARD PRIMER (125ng/ µL)	1 µL

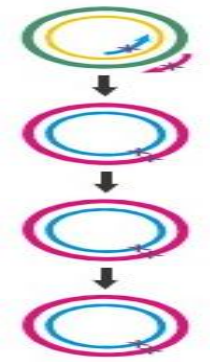
COMPONENTS	VOLUME
REVERSE PRIMER (125ng/ µL)	1 µL
AGILENT Pfu DNA POLYMERASE	0.5 µL
TEMPLATE DNA (100ng/ µL)	0.5 µL
TOTAL VOLUME	25 µL

PCR amplification was conducted under the following thermal cycling conditions:

Table 4.6:- PCR cycling conditions

STEP	TEMPERATURE (°C)	TIME
INITIAL DENATURATION	95°C	30 SECONDS
DENATURATION	95°C	30 SECONDS
ANNEALING	55°C	1 MINUTE
EXTENSION	68°C	8 MINS 20 SECS
HOLD	4°C	∞

16 cycles

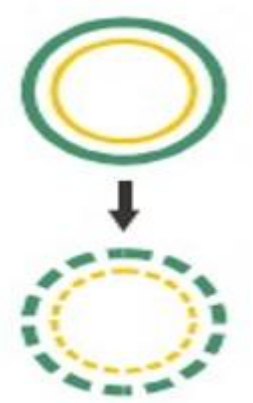


EXTENTION TIME= 1 MIN/KB (8.3 KB)= 8 MIN 20 SECS

NO. OF CYCLES FOR CODON CHANGE= 16

Figure 4.2: Mutagenesis PCR Amplification (~8.3 kb)

4.5. DPN I DIGESTION



After performing the site-directed mutagenesis PCR, I carried out a Dpn I digestion to remove the wild-type (parental) plasmid. The following steps were followed:-

Figure 4.3:- Digestion of the parental plasmid (pCMV Neo Bam p53 wt)

1. Preparation of Reaction Mix

I took 15 µL of the PCR product obtained from the site-directed mutagenesis reaction.

2. Addition of Buffer

To the PCR product, I added 2 µL of 10X Dpn I buffer (provided with the enzyme).

3. Addition of Dpn I Enzyme

I added 1 µL (10 units) of Dpn I enzyme to the mixture. This enzyme specifically digests methylated or hemimethylated DNA.

4. Adjustment of Volume

I added 2 µL of nuclease-free water to bring the total reaction volume to 20 µL.

5. Mixing and Brief Spin

The reaction mixture was gently mixed by pipetting and briefly centrifuged to collect the contents at the bottom of the tube.

6. Incubation

The tube was incubated at 37°C for 1 hour to allow complete digestion of the parental plasmid.

7. Ready for Transformation

The digested product, now enriched for the mutant (unmethylated) plasmid, was directly used for transformation into competent E. coli cells.

The wild-type plasmid used as a template during PCR was isolated from E. coli strains like DH5α, which possess Dam (DNA adenine methyltransferase) activity. This enzyme methylates adenine residues in the GATC sequence. Because of this, the parental plasmid becomes methylated, while the newly synthesized plasmid generated during PCR remains unmethylated, since PCR occurs in vitro without methylating enzymes.

Dpn I recognizes and cleaves methylated GATC sites, enabling it to degrade the parental plasmid without affecting the mutant plasmid. This selective digestion increases the likelihood of successful recovery of clones containing the desired mutation.

4.6. TRANSFORMATION

- Mix 5 µL SDM product with competent cells.
- Ice 10 min → heat shock at 42°C for 1 min → ice 5 min.
- Add 1 mL in LB; recover at 37°C, 45 min (shaking).
- Plate 200 µL on LB agar + ampicillin.
- Incubate overnight at 37°C.

4.7. PLASMID ISOLATION AND RESTRICTION DIGESTION

Plasmid DNA was extracted using a SLS plasmid isolation kit.

Single colonies were picked (Colony no. 2 and Colony no. 12) and inoculated into 5 mL LB broth with ampicillin, followed by overnight incubation at 37°C with shaking at 180 rpm.

4.7.1. PLASMID ISOLATION

- Resuspend bacterial pellet in 250 µL Buffer P1 (with RNase A).
- Add 250 µL Buffer P2, mix gently by inversion.
- Add 350 µL Buffer PN3, mix thoroughly.
- Centrifuge at ≥10,000 rpm for 10 min.
- Transfer supernatant to spin column.
- Centrifuge for 1 min at ≥10,000 rpm; discard flow-through.

- Wash with 0.5 mL Buffer PW1, centrifuge for 1 min at ≥10,000 rpm; discard flow-through.
- Wash with 0.7 mL Buffer PW2, centrifuge for 1 min at ≥10,000 rpm; discard flow through.
- Repeat centrifugation to remove residual wash buffer.
- Elute DNA with 50 µL Buffer PE or water; centrifuge and collect.

Table 4.7:-Concentration of two mutated plasmid from two different colonies

S. No.	Colony No.	Mutated Plasmid	Concentration
1.	2	Isolated Plasmid 1	1.84 µg/µL
2.	12	Isolated Plasmid 2	1.83 µg/µL

Restriction digestion was carried out to confirm the identity of the isolated plasmid and ensure it was the correct construct.

Table 4.8:- Restriction Digestion Reaction Setup for isolated plasmids from two different colonies.

COMPONENTS	VOLUME
PLASMID DNA(Mutated)	2 µL
EcoRI ENZYME	1 µL
10X BUFFER	2 µL
NUCLEASE- FREE WATER	15 µL
TOTAL	20 µL

The reaction mixture was incubated at 37°C for 2 hours in a water bath.

4.8. SEQUENCING PREPARATION

To confirm the presence of the TP53 R248Q mutation in the constructed expression vector, Sanger sequencing was performed. This method is highly reliable for verifying single base pair changes introduced by site-directed mutagenesis.

Sequencing was carried out using the primers specifically designed to flank a 310 bp region containing the mutation site. Both the forward and reverse primers were used in separate reactions to ensure complete and accurate coverage of the target sequence.

To validate the presence and accuracy of the introduced TP53 mutation, primers were designed to amplify a 310 bp region surrounding the mutation site. The reference sequence of the human TP53 gene was retrieved from the NCBI database and analyzed using the Primer-BLAST tool.

Primer-BLAST was used to generate primer pairs with optimal parameters for sequencing, ensuring specificity and amplification efficiency.

Primer pair 9

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TCGACATAGTGTGGTGGTGC	Plus	20	405	424	60.04	55.00	4.00	2.00
Reverse primer	AGAGGAGCTGGTGTTGTTGG	Minus	20	714	695	59.89	55.00	4.00	0.00
Product length	310								

Figure 4.4:- Chosen Primer pair with its characteristics

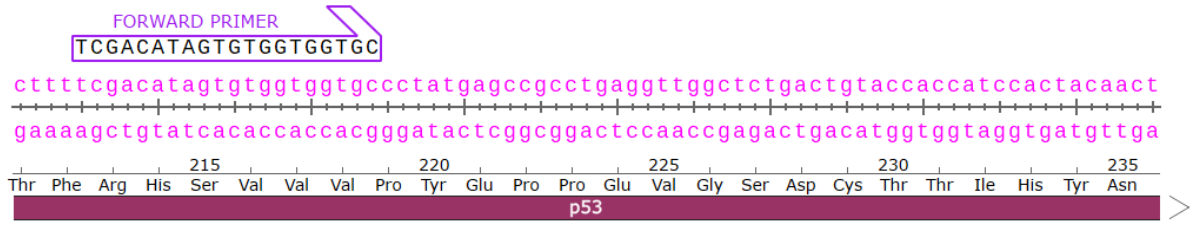


Figure 4.5:- Forward Primer

aagagaatctccgcaagaaaggggagcctcaccacgagctgcccccagggagcactaagcgagcactgcccaaca
ttctcttagaggcgttctttccccctcggagtggtgctcgacgggggtccctcgtgattcgctcgtgacgggttgt
2400
290 295 300 305 310
Glu Glu Asn Leu Arg Lys Lys Gly Glu Pro His His Glu Leu Pro Pro Gly Ser Thr Lys Arg Ala Leu Pro Asn
p53
GGTTGT
REVERSE PRIMER
acaccagctcctctccccagccaaagaagaaaccactggatggagaatatttcacccttcagatccgtgggcgtg
tgtggtcgaggagaggggtcggtttcttctttggtgacctacctcttataaagtgggaagtctaggcacccgcac
2475
315 320 325 330 335
Asn Thr Ser Ser Ser Pro Gln Pro Lys Lys Lys Pro Leu Asp Gly Glu Tyr Phe Thr Leu Gln Ile Arg Gly Arg
p53
TGTGGTCGAGGAGA
REVERSE PRIMER

Figure 4.6:- Reverse primer

Step 1: Preparation of Sequencing Reaction Mixture

For each sequencing reaction (forward and reverse), the following mixture was prepared in a four PCR tubes (Total four tubes) and one with no template.

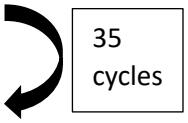
Table 4.9:- Components and their volume for the PCR reaction

COMPONENTS	**VOLUME (µL)**
10X BUFFER	2.5 µL
TAQ POLYMERASE	0.25 µL
FORWARD PRIMER(10 pm/ µL)	0.5 µL
REVERSE PRIMER(10 pm/ µL)	0.5 µL
DNTP MIX	2 µL
`NUCLEASE FREE WATER	14.25 µL
DIGESTED PRODUCT (MUTATED PLASMID)	5 µL
TOTAL	25 µL

Step 2: PCR amplification was conducted under the following thermal cycling conditions:

Table 4.10:- PCR cycling conditions

STEP	TEMPERATURE (°C)	TIME
INITIAL DENATURATION(HOLD)	95°C	5 MINUTES
DENATURATION	95°C	30 SECONDS
ANNEALING	59°C	30 SECONDS
EXTENSION	72°C	30 SECONDS
HOLD 2	72°C	10 MINUTES
HOLD 3	25°C	1 MINUTE



Step 3: Post-PCR Cleanup (SLS PCR Purification Kit)

This procedure was used to purify DNA fragments from PCR reactions using a silica column-based kit.

1. Binding Step: Five volumes of Buffer PW1 were added to one volume of PCR product (e.g., 200 µL PW1 to 50 µL PCR). The mixture was applied to the spin column and centrifuged at ≥10,000 rpm for 1 minute.

2. Wash Step: After discarding the flow-through, 750 µL of Buffer PW2 was added. The column was centrifuged again at ≥10,000 rpm for 1 minute.

3. Second Spin: Flow-through was discarded and the column was spun for another 1 minute to remove ethanol residue.

4. Elution: The column was placed in a clean 1.5 mL tube. DNA was eluted using 50 µL Buffer PE (or 30 µL for higher concentration), then centrifuged at ≥10,000 rpm for 1 minute.

5. Storage: Purified DNA was stored at −20°C until use.

Step 5: Data Analysis

The sequencing chromatograms were viewed and analyzed using Chromas. The obtained sequences were aligned with the reference TP53 gene using SnapGene or online BLAST tools to confirm the presence of the R248Q mutation.

5. RESULTS

5.1. Plasmid Amplification

The parental TP53 plasmid was successfully transformed into E. coli DH5α cells. Transformed colonies were grown in LB broth overnight, and plasmid isolation was performed using a standard alkaline lysis method. Two distinct plasmid samples were obtained, which were confirmed by agarose gel electrophoresis .

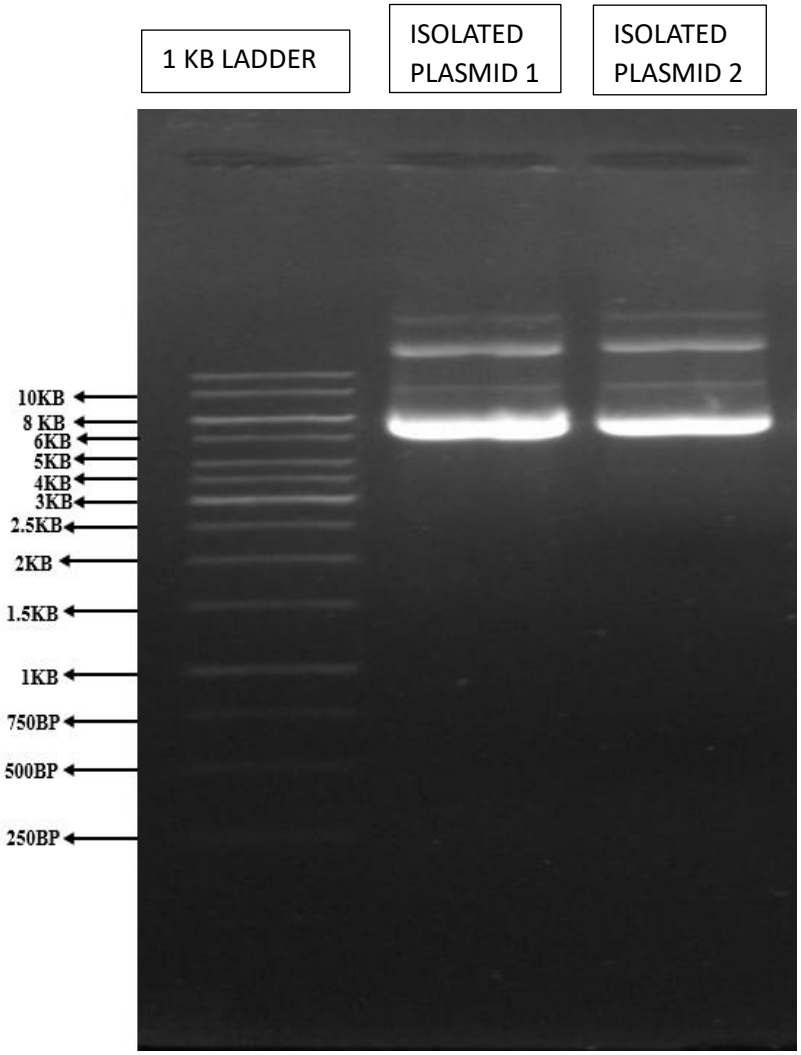


Figure 5.1: Agarose Gel Image of Isolated Plasmids

5.2. Restriction Digestion of Parental Plasmid

The isolated TP53 plasmid was digested using EcoRI, which has three restriction sites in the plasmid. Agarose gel electrophoresis of the digested product showed the expected three-band pattern at approximately 3.6 kb, 2.8 kb, and 1.8 kb, confirming the correct plasmid structure .

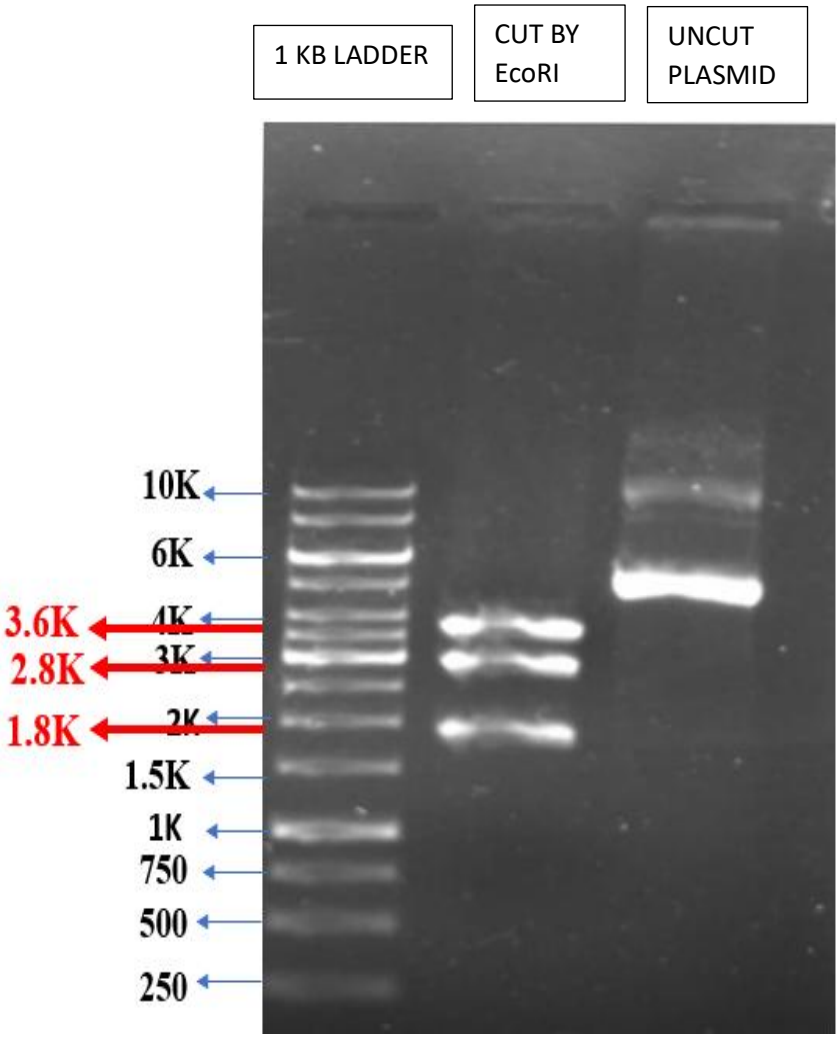


Figure 5.2:- Agarose Gel Image of Restriction Digestion

4.3. Site-Directed Mutagenesis (SDM)

Mutation primers were designed with a GC content of 73% and melting temperature of 72.3°C. Mutagenesis PCR was performed using Agilent Pfu DNA Polymerase under the following conditions: annealing at 55°C, extension at 68°C for 8 minutes 20 seconds for a total of 16 cycles. The DpnI enzyme selectively digested the methylated parental plasmid, enriching the mutant plasmid.

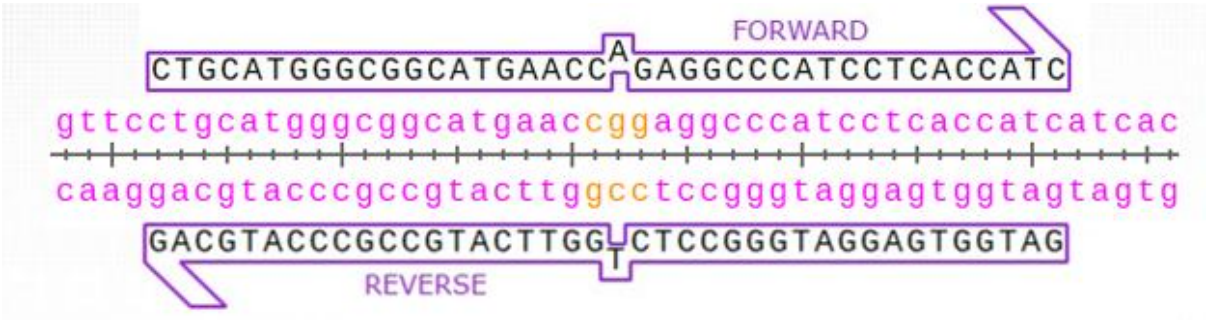


Figure 5.3 :- SDM primers placed in the sequence

4.4. Transformation and Colony Screening

The DpnI-digested PCR product containing the mutated plasmid was transformed into E. coli DH5α. Eighteen colonies were obtained after overnight incubation. Replica plating was performed to preserve colonies for further analysis .

Figure 5.4 : Transformed Colony Growth on Ampicillin Plate

Figure 5.5:- Replica Plate no. 1

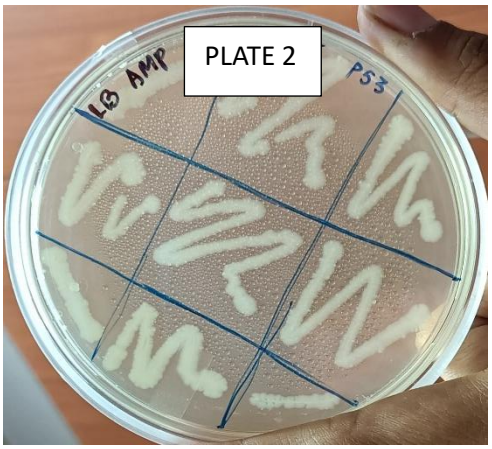


Figure 5.6 :- Replica Plate no. 2

4.5. Confirmation of Mutation by Restriction Digestion

Plasmids were isolated from selected colonies and digested with EcoRI. Gel electrophoresis confirmed the presence of the expected three bands (3.6 kb, 2.8 kb, and 1.8 kb), indicating the presence of the complete plasmid with insert .

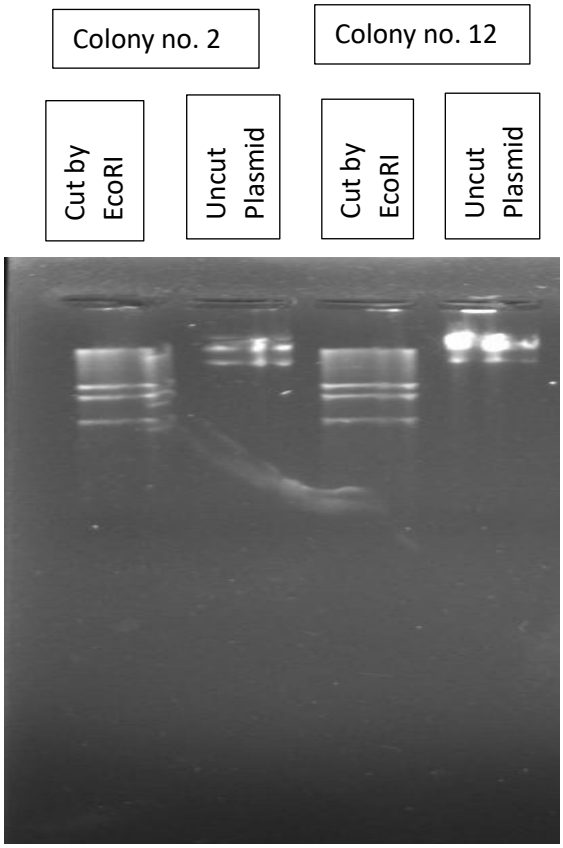


Figure 5.7:- Restriction Digestion of the mutated plasmid

4.6. Sequencing Confirmation

PCR was performed using mutation-specific primers, yielding a 310 bp product. The PCR product was purified and subjected to sequencing. Comparison of pre- and post-mutagenesis sequences confirmed the presence of the R248Q mutation (CGG → CAG) in the TP53 gene.

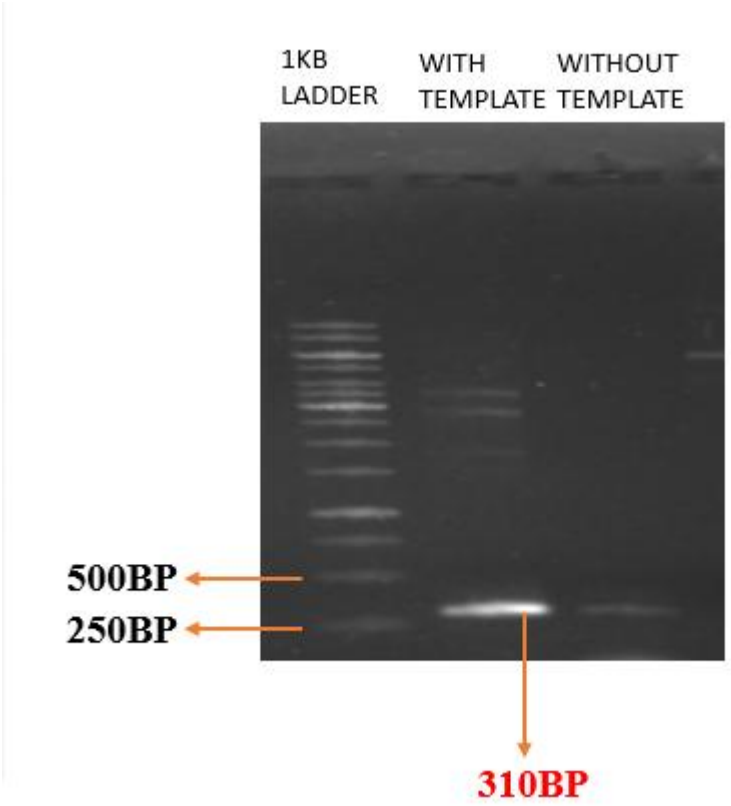


Figure 5.8:- PCR standardisation by the sequencing primers.

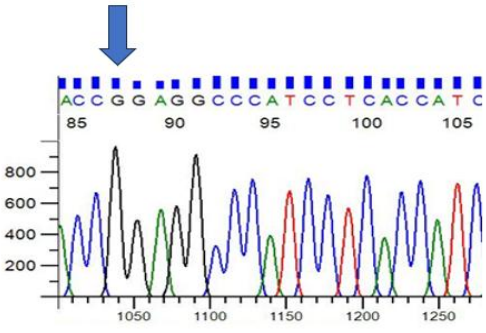


Figure 5.9:- Before Mutation

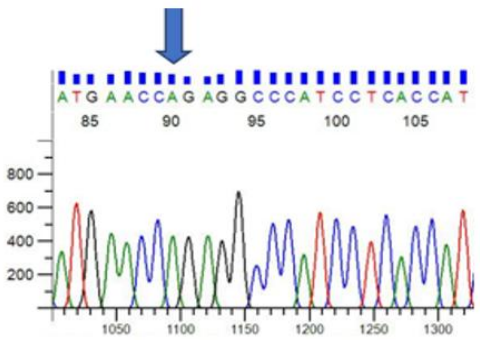


Figure 5.10:- After Mutation

6. DISCUSSION

This study successfully generated a mammalian expression vector containing the R248Q mutation in the TP53 gene using site-directed mutagenesis. The mutation was introduced accurately and confirmed through sequencing, indicating that the mutagenesis and cloning steps were effective. This lays the groundwork for future studies aimed at exploring how this specific mutation alters p53 function in mammalian cells.

The R248Q mutation is one of the most frequently reported hotspot mutations in TP53 and is found in a wide range of human cancers. It affects the DNA-binding domain of p53, a region essential for its tumor-suppressing activity. By disrupting this domain, the R248Q variant is known to impair the ability of p53 to regulate target genes involved in controlling cell growth, inducing apoptosis, and maintaining genomic integrity. Constructing an expression vector that carries this mutation allows for its functional study in controlled cellular environments, helping to better understand how it contributes to cancer progression.

Introducing the mutant gene into a suitable vector makes it possible to express the altered p53 protein in mammalian cells. This opens up opportunities to examine its biological effects, such as changes in cell proliferation, resistance to cell death, and altered responses to DNA damage. Previous research has shown that the R248Q mutation can result in both a loss of tumor suppressor function and, in some cases, gain-of-function properties that promote tumor growth and invasion. Having a reliable model to study these effects will be valuable in identifying potential therapeutic targets.

While the vector construction was successful, there are a few limitations to acknowledge. This project focused on building and confirming the vector, but did not include functional assays to test the behavior of the mutant protein. Future work should involve transfecting the vector into mammalian cells and evaluating changes in gene expression, cell cycle dynamics, and other phenotypic effects. Additionally, although sequencing confirmed the presence of the R248Q mutation, a full plasmid sequence would be necessary to ensure no unintended changes occurred during cloning.

Overall, this project achieved its goal of constructing a TP53 R248Q expression vector, which will serve as a valuable tool for further research. It contributes to the growing body of work aimed at understanding how specific TP53 mutations influence cancer development and progression and provides a foundation for exploring targeted treatments in the future.

7. CONCLUSION

This research successfully achieved the construction of a mammalian expression vector carrying the TP53 R248Q mutation through site-directed mutagenesis. The process was carefully carried out, and sequencing confirmed that the intended mutation was accurately introduced. This outcome marks an important step toward enabling more detailed studies on how this specific mutation affects the function of the p53 protein.

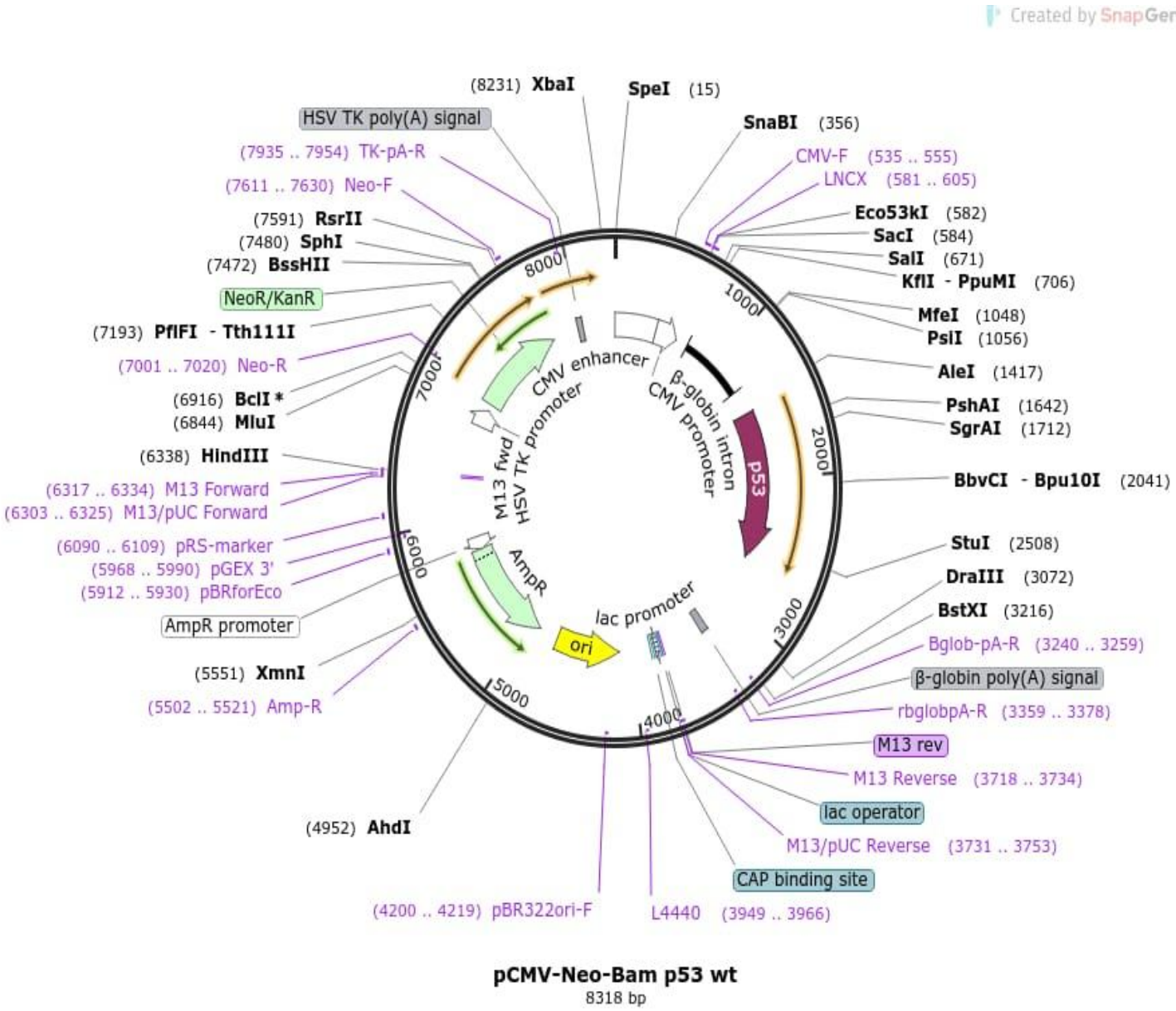
The R248Q mutation is widely known for its involvement in various types of cancer, primarily due to its location in the DNA-binding domain of p53—a region critical for its tumor-suppressive role. By creating a tool that allows controlled expression of this mutant protein in mammalian cells, this study helps pave the way for functional investigations that could deepen our understanding of p53-related cancer biology.

Although this project focused on the vector construction and verification, it lays the foundation for future work. The next logical step would be to study the biological effects of the R248Q mutation in cell-based models—looking at how it might influence cell growth, survival, or response to stress. These insights could eventually support the development of more targeted approaches for cancer treatment.

By providing a functional vector with a known cancer-associated TP53 mutation, it offers a valuable resource for future research aimed at exploring the role of mutant p53 and identifying potential therapeutic strategies.

8. APPENDIX

8.1. PLASMID MAP OF pCMV–Neo–Bam p53 wt



pCMV-Neo-Bam p53 wt
8318 bp

8.2 SEQUENCING PREPARATION PRIMER DESIGNING REGION

caccatgagcgctgctcagatagcgatggtctggcccctcctcagcatcttatccgagtggaaggaaatttgcgtgtggagtatttggatgacagaaaca
gtggtactcgcgacgagtctatcgctaccagaccggggaggagtcgtagaataggctcaccttcctttaaacgcacacctcataaacctactgtctttgt
180 185 190 195 200 205 210
His His Glu Arg Cys Ser Asp Ser Asp Gly Leu Ala Pro Pro Gln His Leu Ile Arg Val Glu Gly Asn Leu Arg Val Glu Tyr Leu Asp Asp Arg Asn
p53
ctttcgacatagtgtggtggtgccctatgagccgcctgaggttggctctgactgtaccaccatccactacaactacatgtgtaacagttcctgcatggg
gaaaagctgtatcacaccaccacgggatactcggcggactccaaccgagactgacatggtggtaggtgatgttgatgtacacattgtcaaggacgtaccc
215 220 225 230 235 240
Thr Phe Arg His Ser Val Val Val Pro Tyr Glu Pro Pro Glu Val Gly Ser Asp Cys Thr Thr Ile His Tyr Asn Tyr Met Cys Asn Ser Ser Cys Met Gly
p53
cggcatgaaccggaggcccatcctcaccatcatcacactggaagactccagtggtaatctactgggacggaacagctttgaggtgcgtgtttgtgcctgt
gccgtacttggcctccgggtaggagtggtagtagtgtgaccttctgaggtcaccattagatgaccctgccttgtcgaaactccacgcacaaacacggaca
245 250 255 260 265 270 275
Gly Met Asn Arg Arg Pro Ile Leu Thr Ile Ile Thr Leu Glu Asp Ser Ser Gly Asn Leu Leu Gly Arg Asn Ser Phe Glu Val Arg Val Cys Ala Cys
p53
cctgggagagaccggcgcacagaggaagagaatctccgcaagaaaggggagcctcaccacgagctgcccccagggagcactaagcgagcactgcccaaca
ggaccctctctggccgcgtgtctccttctcttagaggcgttctttcccctcggagtggtgctcgacgggggtccctcgtgattcgctcgtgacgggttgt
280 285 290 295 300 305 310
Pro Gly Arg Asp Arg Arg Thr Glu Glu Glu Asn Leu Arg Lys Lys Gly Glu Pro His His Glu Leu Pro Pro Gly Ser Thr Lys Arg Ala Leu Pro Asn
p53

9. REFERENCES

A high-efficiency method for site-directed mutagenesis of large plasmids based on large DNA fragment amplification and recombinational ligation

https://www.nature.com/articles/s41598-021-89884-z

A Simple and Economical Site-Directed Mutagenesis Method for Large Plasmids by Direct Transformation of two Overlapping PCR Fragments

https://www.tandfonline.com/doi/full/10.2144/btn-2022-0085

DOI:- %20https:/doi.org/10.2144/btn-2022-0085

An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol

https://bmcbiotechnol.biomedcentral.com/articles/10.1186/1472-6750-8-91

Enhancement of the antiproliferative function of p53 by phosphorylation at serine 20: an inference from site-directed mutagenesis studies

https://pubmed.ncbi.nlm.nih.gov/11172619/

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High Efficiency of Site-Directed Mutagenesis Mediated by a Single PCR Product

https://academic.oup.com/nar/article/25/3/682/2361249

DOI: https://doi.org/10.1093/nar/25.3.682

Rapid and efficient site-specific mutagenesis without phenotypic selection

https://www.pnas.org/content/82/2/488

REPLACR-mutagenesis, a one-step method for site-directed mutagenesis by recombineering

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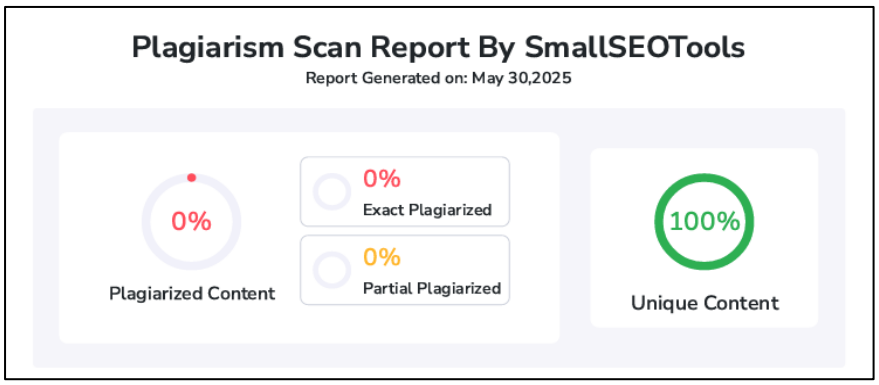
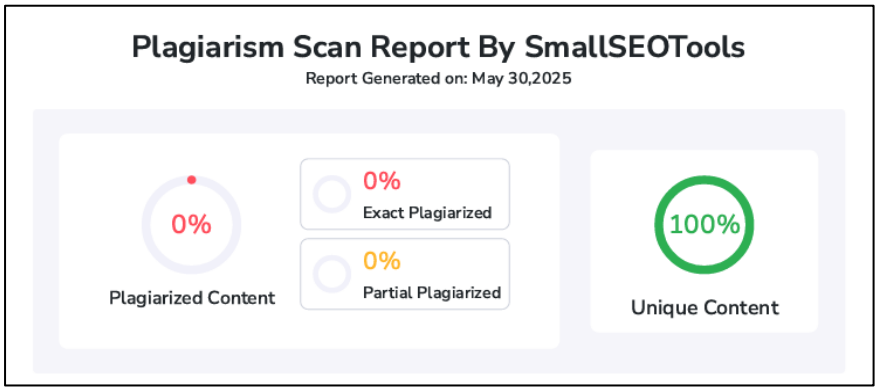
DOI: 10.1089/109065702761403333
Suppression of human colorectal carcinoma cell growth by wild-type p53
Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B
Science. 1990 Aug 24; 249(4971):912-5.

10. PLAGARISM REPORT

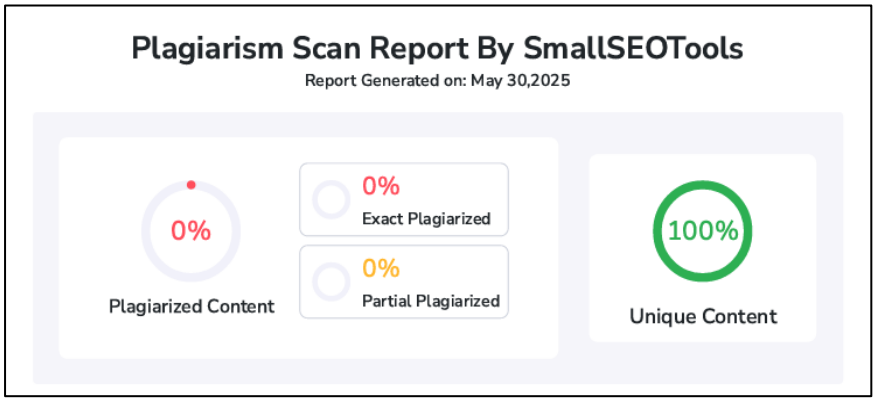
10.1 ABSTRACT



10.2 INTRODUCTION



10.3 REVIEW OF LITRATURE



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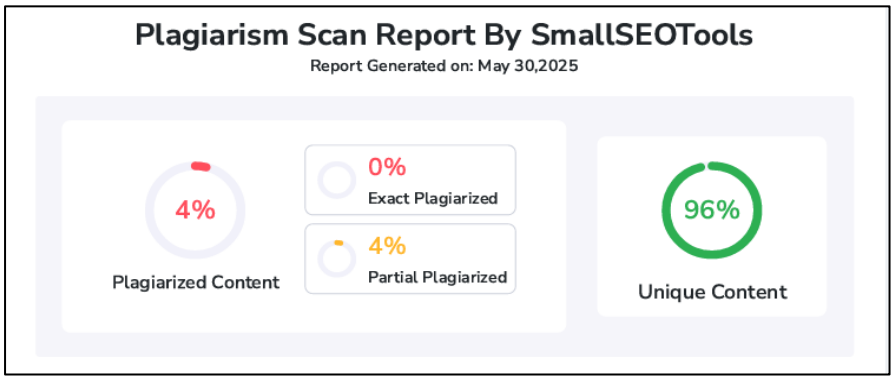

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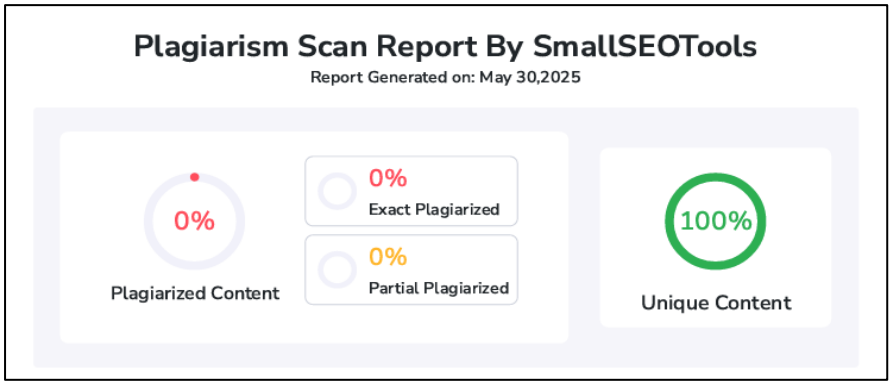
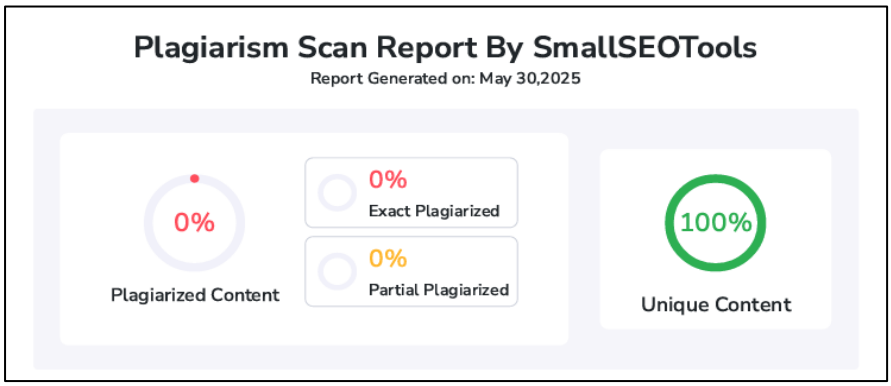


10.6 CONCLUSION



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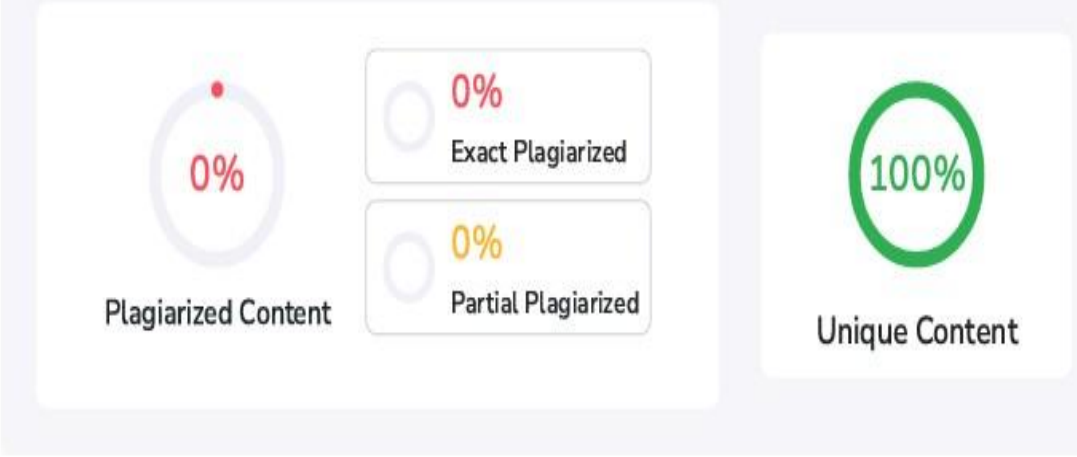

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ABSTRACT

The TP53 gene is one of the most studied tumor suppressors due to its vital role in controlling cell growth and preserving genomic integrity. Among the many mutations reported in cancers, the R248Q substitution is notably recurrent and functionally significant. This project focused on introducing the R248Q mutation into a mammalian expression vector containing the wild-type TP53 gene. The mutation was successfully incorporated and validated, resulting in a plasmid construct that mimics a common cancer-associated alteration. The study highlights the practical application of molecular techniques to modify specific genetic sequences with precision. While the experimental process involved careful planning and verification, the outcome reflects a straightforward and effective strategy to model known mutations at the molecular level. The generated construct offers a representation of how a single nucleotide change can impact a gene known for its protective role in human cells. This work adds to the understanding of TP53 mutation modeling and reflects the importance of detailed molecular research in uncovering the complexities of gene function and alteration.

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1. INTRODUCTION

Site-directed mutagenesis (SDM) is a powerful molecular biology technique that enables the precise alteration of specific DNA sequences at predetermined locations. This method allows researchers to introduce targeted mutations such as point mutations, insertions, or deletions into a gene of interest, facilitating the study of gene function, protein structure, and interactions. SDM has become an indispensable tool in genetic engineering, protein engineering, and functional genomics, providing insights into the molecular mechanisms underlying various biological processes and diseases.
Located on chromosome 17p13.1, the TP53 gene encodes the p53 protein, a vital tumor suppressor that plays a key role in preserving genomic stability .It manages the cell's response to DNA damage by controlling processes such as cell cycle arrest, DNA repair, programmed cell death, and cellular aging. Due to its pivotal function in controlling cell proliferation and preventing malignant transformation, TP53 is often referred to as the "guardian of the genome." Alterations in the TP53 gene are among the most prevalent genetic abnormalities in human cancers, detected in more than half of all tumor types. These mutations frequently lead to loss of normal p53 function, allowing cells with damaged DNA to evade growth arrest and apoptosis, thereby promoting tumorigenesis.

Among the numerous mutations identified in TP53, the R248Q mutation is a prominent hotspot mutation located within the DNA-binding domain of the p53 protein. This mutation results from a single nucleotide substitution, changing the codon CGG (arginine) to CAG (glutamine) at position 248. Arginine at position 248 plays a critical role in DNA binding, and its substitution severely disrupts p53's capacity to attach to DNA and initiate transcription of its target genes. The R248Q mutation is associated with dominant-negative effects over wild-type p53 and gain-of-function oncogenic properties, contributing to cancer progression, metastasis, and resistance to chemotherapy.

Given the biological and clinical significance of the R248Q mutation, constructing a mammalian expression vector harboring this specific mutation is essential for functional studies. Such a vector allows for the expression of mutant p53 in mammalian cells, enabling detailed investigation of its effects on cellular pathways, tumorigenic potential, and response to therapeutic agents. Site-directed mutagenesis provides a reliable and efficient approach to introduce the R248Q mutation into the TP53 gene within a plasmid vector, facilitating downstream functional assays.

This study aims to construct a mammalian expression vector containing the TP53 R248Q mutation by employing PCR-based site-directed mutagenesis. The parental wild-type TP53 plasmid will be amplified in Escherichia coli DH5α cells, followed by mutagenesis PCR using specifically designed primers to introduce the desired codon change. Confirmation of successful mutagenesis will be performed through restriction enzyme digestion and DNA sequencing. The resulting mutant vector will

serve as a valuable tool for further research into the molecular mechanisms of mutant p53-driven cancers and the development of targeted therapies.

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2. REVIEW OF LITRATURE

Advances in molecular cloning and gene editing have made it possible to introduce specific mutations into mammalian genes, providing powerful tools for studying the molecular basis of diseases such as cancer. One of the most impactful applications of these technologies is the construction of mammalian expression vectors carrying clinically relevant mutations, such as those found in the TP53 tumor suppressor gene. This project focuses on the TP53 gene, which encodes the p53 protein—a central player in tumor suppression—and utilizes site-directed mutagenesis to introduce a clinically significant mutation.

TP53, located on chromosome 17p13.1, is widely regarded as the "guardian of the genome." The p53 protein it encodes acts as a transcription factor that regulates genes involved in cell cycle arrest, apoptosis, DNA repair, and senescence. Under normal conditions, p53 remains at low levels due to rapid degradation, but upon cellular stress or DNA damage, it accumulates in the nucleus and initiates protective responses. This critical function is underscored by the fact that TP53 is the most frequently mutated gene in human cancers, with mutations observed in over half of all tumors (Olivier et al., 2010).

The p53 protein consists of several domains: an N-terminal transactivation domain, a central DNA-binding domain, and a C-terminal tetramerization domain. The DNA-binding domain is particularly important, as it allows p53 to recognize and bind to specific DNA sequences, thereby controlling the expression of downstream genes. Most cancer-associated mutations, including the R248Q mutation studied here, occur within this domain and disrupt p53's ability to bind DNA and regulate gene expression.

Missense mutations in TP53 are the most common type found in cancers. These mutations often result in the production of full-length but dysfunctional proteins. Some mutant forms, such as R248Q, not only lose their tumor-suppressive function but also gain new oncogenic properties—promoting cell survival, proliferation, and resistance to therapy (Brosh & Rotter, 2009). The R248Q mutation specifically changes an arginine to a glutamine at position 248, a residue that directly contacts DNA. This alteration severely impairs p53's DNA-binding ability and is associated with aggressive tumor phenotypes (Cho et al., 1994).

Site-directed mutagenesis (SDM) is a cornerstone technique in molecular biology that enables targeted changes to DNA sequences. By designing primers that introduce specific nucleotide substitutions, researchers can recreate disease-associated mutations or study protein structure-function relationships. The PCR-based SDM method is widely used for its efficiency and precision. Following amplification, DpnI digestion is used to selectively degrade the methylated parental DNA, thereby enriching the sample for the newly synthesized mutant plasmid (Zheng et al., 2004). SDM has been instrumental in generating mutant constructs for functional studies, drug screening, and therapeutic development.

The construction of a mammalian expression vector carrying a specific TP53 mutation involves several key steps. First, the wild-type TP53 gene is cloned into a suitable plasmid backbone. In this project, mutagenic primers were designed to introduce the R248Q mutation (CGG to CAG) during PCR amplification. The amplified product is treated with DpnI to digest the original template, and the mutated plasmid is then transformed into competent E. coli cells for propagation.

Verification of the construct is essential. Plasmid DNA is isolated from bacterial colonies and analyzed by restriction digestion—using enzymes such as EcoRI—to confirm the presence and integrity of the insert. Sequencing is performed to ensure the correct mutation has been introduced and that no unintended changes have occurred. These validation steps are critical before proceeding to functional assays in mammalian cells.

Mammalian expression vectors play a crucial role in modern biomedical research by enabling scientists to express specific genes in a controlled and physiologically relevant environment. Unlike bacterial systems, mammalian cells provide the necessary post-translational modifications, folding mechanisms, and cellular context required for human proteins to function accurately. This is particularly important when studying complex regulatory proteins like p53, whose activity depends heavily on its interactions with other cellular components and its regulation through phosphorylation, acetylation, and other modifications. By using mammalian expression vectors, researchers can more faithfully replicate the conditions found in human tissues, allowing for more accurate insights into gene function, disease mechanisms, and potential therapeutic responses. These vectors not only facilitate the study of mutant proteins like p53-R248Q but also serve as platforms for testing drug candidates, developing gene therapies, and tailoring treatments to individual genetic profiles—bringing us closer to truly personalized medicine.

Mutant p53 constructs, such as those harboring the R248Q mutation, are invaluable for dissecting the molecular mechanisms of tumorigenesis. By expressing these constructs in mammalian cells, researchers can study the effects of specific mutations on cell cycle regulation, apoptosis, and response to DNA damage. These models also provide platforms for screening drugs that restore wild-type p53 function or selectively target cells with mutant p53.

Furthermore, understanding the behavior of mutant p53 proteins has direct clinical implications. For example, certain p53 mutations are associated with resistance to chemotherapy, influencing treatment strategies and patient outcomes. The knowledge gained from these studies supports the development of personalized medicine approaches in oncology.

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4. METHADOLOGY

4.1. PLASMID ISOLATION

Amplifying the parental plasmid DNA (pCMV Neo Bam p53 wt) to obtain sufficient quantities for downstream applications such as SITE DIRECTED MUTAGENESIS, SEQUENCING, etc.

4.1.1. COMPETENT CELL PREPARATION

1. Primary Culture:

• Inoculate a single DH5α colony into 5 mL LB broth.

• Incubate overnight at 37°C (130 rpm).

2. Secondary Culture:

• Transfer 500 µL of primary culture into 5 mL fresh LB broth.

• Incubate at 37°C for ~1.5 hours (235 rpm) until OD600 reaches 0.4–0.6.

3. Competent Cell Preparation:

• Centrifuge 1.5 mL of the bacterial culture (in duplicate) at 10,000 rpm for 1 minute to pellet the cells.

• Resuspend in 500 µL cold transformation buffer. Chill on ice 15 min.

• Pellet again; resuspend in 200 µL cold transformation buffer.

4.1.2. TRANSFORMATION

• Add 2 µL of the pCMV Neo Bam p53 wild-type plasmid to the competent cells and mix gently..

• Ice 10 min → heat shock at 42°C for 90 sec → ice 5 min.

• Add 800 µL LB; recover at 37°C, 45 min (shaking).

• Plate 200 µL on LB agar + ampicillin.

• Incubate overnight at 37°C.

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4.1.3. PLASMID ISOLATION

1 Firstly, suspend the bacterial pellet in 250 µL of Buffer P1 that contains RNase A. 2. Add 250 µL Buffer P2, mix gently by inversion.

3. Add 350 µL Buffer PN3, mix thoroughly.

4. Centrifuge at ≥10,000 rpm for 10 min.

5. Transfer supernatant to spin column.

6. Centrifuge the given sample at 10,000 rpm or more for 1 minute straight, then discard the liquid flow-through.
7. Add 0.5 mL of Buffer PW1 to wash the column then spin the column again, and gently remove the flow-through.
8. Wash with 0.7 mL Buffer PW2, centrifuge.
9. Repeat centrifugation to remove residual wash buffer.
10. Elute DNA with 50 µL nuclease free water; centrifuge and collect.

4.1.4. AGAROSE GEL ELECTROPHORESIS

A 1% agarose gel was prepared by dissolving 0.3 g of agarose in 30 mL of 1X TAE buffer. After cooling the molten agarose to approximately 60°C, Ethidium Bromide was added to a final concentration of 1X after partially cooling it down. The gel was cast in a tray with a comb and allowed to solidify.

The isolated plasmid sample (6 µL) was mixed with 1 µL of 6X loading dye and loaded into the well alongside a 1 kb DNA ladder. Electrophoresis was performed in 1X TAE buffer at 80 V for 60 minutes. The gel was then visualized under a UV transilluminator, and the image was captured using a gel documentation system.

Table 4.1 :- Concentrations of pCMV- Neo-Bam p53 wt plasmid

Sl.No.	pCMV Neo Bam p53 wt	Concentration
1.	Isolated plasmid (1)	2.14 µg/µL
2.	Isolated plasmid (2)	2.30 µg/µL

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4.2. RESTRICTION DIGESTION OF PLASMID

To confirm the identity of the amplified pCMV-Neo-Bam p53 wt plasmid by restriction digestion using the EcoRI enzyme, which cuts at three known sites and yields three distinct DNA fragments.

Table 4.2: Restriction Digestion Reaction Setup

COMPONENTS	VOLUME
PLASMID DNA(2.30 µg/µL)	2 µL
EcoRI ENZYME	1 µL
10X BUFFER	2 µL
NUCLEASE- FREE WATER	15 µL
TOTAL	20 µL

The reaction mixture was incubated at 37°C for 2 hours in a water bath.

4.2.1. AGAROSE GEL ELECTROPHORESIS

A 1% agarose gel was prepared by dissolving 0.3 g of agarose in 30 mL of 1X TAE buffer. After cooling the molten agarose to approximately 60°C, Ethidium Bromide was added to a final concentration of 1X after partially cooling it down. The gel was cast in a tray with a comb and allowed to solidify.

The digested plasmid sample (10 µL) was mixed with 2 µL of 6X loading dye and loaded into the well alongside a 1 kb DNA ladder. Electrophoresis was performed in 1X TAE buffer at 80 V for 60 minutes. The gel was then visualized under a UV transilluminator, and the image was captured using a gel documentation system.

Expected Bands: ~3.6 kb, ~2.8 kb, and ~1.8 kb

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4.3. SITE-DIRECTED MUTAGENESIS PRIMER DESIGNING

The objective of this step was to design mutagenic primers for the introduction of a specific point mutation—R248Q (CGG → CAG)—in the TP53 gene using a PCR-based site-directed mutagenesis strategy. At codon 248 of the TP53 protein, this mutation causes arginine (R) to be substituted by glutamine (Q).

Step 1: Identification of the Mutation Site

Figure 4.1:- Mutation site in the p53 sequence

The codon for arginine at position 248 (CGG) was identified within the TP53 sequence. The corresponding nucleotide sequence in the plasmid was located using SnapGene.

Step 2: Determination of Desired Codon Change

At codon 248 of the TP53 protein, this mutation causes arginine (R) to be substituted by glutamine (Q).

Step 3: Extraction of Flanking Sequences

Exact 19 nucleotides upstream and downstream of the target nucleotide were extracted to ensure appropriate primer binding during PCR amplification.

Step 4: Primer Design

The mutation was centrally located within the forward primer, and the reverse primer was the exact reverse complement. Both primers were between 39 bp in length, with GC content 63% , and a Tm 72.3°C, suitable for high-fidelity PCR.

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Table 4.3:- Information about Forward and Reverse Primer.

FORWARD PRIMER	REVERSE PRIMER
5'- CTGCATGGGCGGCATGAACCAGAGG CCCATCCTCACCATC-3'	5'- GATGGTGAGGATGGGCCTCTGGTTCA TGCCGCCCATGCAG-3'
GC CONTENT :- 63%	GC CONTENT :- 63%
Tm 72.3°C	Tm 72.3°C
LENGTH:- 39 bp	LENGTH:- 39 bp

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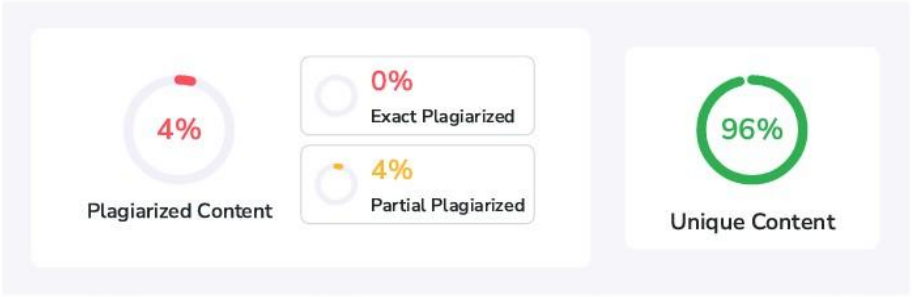

Total Words: 842 **Total Characters:** 5205 **Plagiarized Sentences:** 1.72 **Unique Sentences:** 41.28 (96%)

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4.4. MUTAGENESIS PCR

The PCR was carried out using Pfu DNA polymerase, known for its proofreading activity and high-fidelity amplification, to minimize errors during synthesis. The final volume of the reaction resulted 25 µL. Each reaction mixture contained 50 ng of plasmid DNA (wild-type TP53), 1 µL each of forward and reverse primers (125ng), 2 µL of dNTP mix (25 mM each), 2.5 µL of 10X high-fidelity buffer, and 0.5 µL of Pfu DNA polymerase. After that, volume adjusted to 25 µL by nuclease free water.

Table 4.4:- Stock and final concentrations of three components

S.No. COMPONENTS STOCK CONCENTRATION FINAL CONCENTRATION

1. Forward Primer 1.22 µg/µL 1.25 µg/µL
2. Reverse Primer 1.39 µg/µL 1.25 µg/µL
3. Template DNA 2.30 µg/µL 1.00 µg/µL

Table 4.5:-Components and their volume for Site Directed Mutagenesis PCR.

COMPONENTS VOLUME

NUCLEASE FREE WATER 17.5 µL
AGILENT 10X BUFFER 2.5 µL
DNTP (24Mm) 2 µL
FORWARD PRIMER (125ng/ µL) 1 µL
REVERSE PRIMER (125ng/ µL) 1 µL
AGILENT Pfu DNA POLYMERASE 0.5 µL
TEMPLATE DNA (100ng/ µL) 0.5 µL
TOTAL VOLUME 25 µL

Table 4.6:- PCR cycling conditions

STEP TEMPERATURE (°C) TIME

INITIAL DENATURATION 95°C 30 SECONDS
DENATURATION 95°C 30 SECONDS
ANNEALING 55°C 1 MINUTE
EXTENSION 68°C 8 MINS 20 SECS
HOLD 4°C

Figure 4.2: Mutagenesis PCR Amplification (~8.3 kb)

4.5. DPN I DIGESTION

After performing the site-directed mutagenesis PCR, I carried out a Dpn I digestion to remove the wild-type (parental) plasmid. The following steps were followed:-

1. Preparation of Reaction Mix

I took 15 µL of the PCR product obtained from the site-directed mutagenesis reaction.

2. Addition of Buffer

To the PCR product, I added 2 µL of 10X Dpn I buffer (provided with the enzyme).

3. Addition of Dpn I Enzyme

Then, I added 1 µL of Dpn I enzyme to the mixture. This enzyme specifically digests methylated or hemimethylated DNA.

4. Adjustment of Volume

I added 2 µL of nuclease-free water to bring the total reaction volume to 20 µL.

5. Mixing and Brief Spin

The mixture was then gently mixed by pipetting followed by centrifugation to collect the contents present at the bottom of the tube.

6. Incubation

The tube was then incubated at 37°C for 1 hour to allow complete digestion of the pCMV Neo Bam p53 wt

7. Ready for Transformation

The digested product, now enriched for the mutant (unmethylated) plasmid, was directly used for transformation into competent E. coli cells.

The wild-type plasmid used as a template during PCR was isolated from E. coli strains like DH5α, which possess Dam (DNA adenine methyltransferase) activity. Methylation of adenine residues is done at GATC sequence. Because of this, the parental plasmid becomes methylated, while the newly synthesized plasmid generated during PCR remains unmethylated, since PCR occurs in vitro without methylating enzymes.

Dpn I recognizes and cleaves methylated GATC sites, enabling it to degrade the parental plasmid without affecting the mutant plasmid. This selective digestion increases the likelihood of successful recovery of clones containing the desired mutation.

4.6. TRANSFORMATION

- Combine 5 µL of the site-directed mutagenesis (SDM) product with competent cells.

Incubate the mixture on ice for 10 minutes, followed by heat shock at 42°C for 1 minute, then return to ice for 5 minutes.. Add 1 mL of LB medium to the cells and incubate at 37°C for 45 minutes with shaking for recovery.

- Plate 200 µL on LB agar + ampicillin.
- Incubate overnight at 37°C.

4.7. PLASMID ISOLATION AND RESTRICTION DIGESTION

Plasmid DNA was isolated using the SLS plasmid extraction kit.

Individual colonies (specifically colony 2 and colony 12) were selected and cultured in 5 mL of LB broth containing ampicillin, then incubated overnight at 37°C with shaking at 180 rpm.

4.7.1. PLASMID ISOLATION

Resuspend the bacterial pellet in 250 µL of Buffer P1 containing RNase A.

Add 250 µL of Buffer P2 and gently mix by inverting the tube.

Add 350 µL of Buffer PN3 and mix thoroughly to neutralize the solution.

Centrifuge the mixture at 10,000 rpm or higher for 10 minutes to pellet cellular debris..

- Transfer supernatant to spin column.
- Centrifuge for 1 min at ≥10,000 rpm; discard flow-through.
- Wash with 0.5 mL Buffer PW1, centrifuge for 1 min at ≥10,000 rpm; discard flow-through.
- Wash with 0.7 mL Buffer PW2, centrifuge for 1 min at ≥10,000 rpm; discard flow through.

• Repeat centrifugation to remove residual wash buffer.
• Elute DNA with 50 µL Buffer PE or water; centrifuge and collect.

Table 4.7:-Concentration of two mutated plasmid from two different colonies

S. No.	Colony No.	Mutated Plasmid	Concentration
1.	2	Isolated Plasmid 1	1.84 µg/µL
2.	12	Isolated Plasmid 2	1.83 µg/µL

Restriction digestion was carried out to confirm the identity of the isolated plasmid and ensure it was the correct construct.

Table 4.8:- Restriction Digestion Reaction Setup for isolated plasmids from two different colonies.

COMPONENTS	VOLUME
PLASMID DNA(Mutated)	2 µL
EcoRI ENZYME	1 µL
10X BUFFER	2 µL
NUCLEASE- FREE WATER	15 µL
TOTAL	20 µL

The reaction mixture was incubated at 37°C for 2 hours in a water bath.

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4.8. SEQUENCING PREPARATION
To confirm the presence of the TP53 R248Q mutation in the constructed expression vector, Sanger sequencing was performed. This method is highly reliable for verifying single base pair changes introduced by site-directed mutagenesis.

Sequencing was carried out using the primers specifically designed to flank a 310 bp region containing the mutation site. The reference sequence of
the human TP53 gene was retrieved from the NCBI database and analyzed using the Primer BLAST tool.
Primer-BLAST was used to generate primer pairs with optimal parameters for sequencing, ensuring specificity and amplification efficiency.

Figure 4.4:- Chosen Primer pair with its characteristics
Figure 4.5:- Forward Primer

Figure 4.6:- Reverse primer
Step 1: Preparation of Sequencing Reaction Mixture
For each sequencing reaction (forward and reverse)

Step 2: PCR amplification was conducted under the following thermal cycling conditions:

COMPONENTS VOLUME (µL)
10X BUFFER 2.5 µL
TAQ POLYMERASE 0.25 µL
FORWARD PRIMER(10 pm/ µL) 0.5 µL
REVERSE PRIMER(10 pm/ µL) 0.5 µL
DNTP MIX 2 µL
` NUCLEASE FREE WATER 14.25 µL
DIGESTED PRODUCT (MUTATED

PLASMID)
5 µL
TOTAL 25 µL
Table 4.10:- PCR cycling conditions
STEP TEMPERATURE (°C) TIME
INITIAL
DENATURATION(HOLD)
95°C 5 MINUTES
DENATURATION 95°C 30 SECONDS
ANNEALING 59°C 30 SECONDS
EXTENSION 72°C 30 SECONDS
HOLD 2 72°C 10 MINUTES
HOLD 3 25°C 1 MINUTE

Step 3: Post-PCR Cleanup (SLS PCR Purification Kit)
This procedure was used to purify DNA fragments from PCR reactions using a silica column based kit.
1. Binding Step: Five volumes of Buffer PW1 were added to one volume of PCR product (e.g., 200 µL PW1 to 50 µL PCR).
2. Wash Step: After discarding the flow-through, 750 µL of Buffer PW2 was added. The column was centrifuged again at ≥10,000 rpm for 1 minute.
3. Second Spin: Flow-through was discarded and the column was spun for another 1 minute to remove ethanol residue.
4. Elution: The column was placed in a clean 1.5 mL tube. DNA was eluted using 50 µL Buffer PE (or 30 µL for higher concentration), then centrifuged at ≥10,000 rpm for 1 minute.
5. Storage: Purified DNA was stored at −20°C until use.
Step 5: Data Analysis
The sequencing chromatograms were viewed and analyzed using Chromas. The obtained sequences were aligned with the reference TP53 gene using SnapGene or online BLAST tools to confirm the presence of the R248Q mutation.

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5.1. Plasmid Amplification

The parental TP53 plasmid was successfully transformed into E. coli DH5α cells. Transformed colonies were grown in LB broth overnight, and plasmid isolation was performed using a standard alkaline lysis method. Two distinct plasmid samples were obtained, which were confirmed by agarose gel electrophoresis .

Figure 5.1: Agarose Gel Image of Isolated Plasmids

5.2. Restriction Digestion of Parental Plasmid

The isolated TP53 plasmid was digested using EcoRI, which has three restriction sites in the plasmid. Agarose gel electrophoresis of the digested product showed the expected three-band pattern at approximately 3.6 kb, 2.8 kb, and 1.8 kb, confirming the correct plasmid structure .

Figure 5.2:- Agarose Gel Image of Restriction Digestion

4.3. Site-Directed Mutagenesis (SDM)

Mutation primers were designed with a GC content of 73% and melting temperature of 72.3°C. Mutagenesis PCR was performed using Agilent Pfu DNA Polymerase under the following conditions: annealing at 55°C, extension at 68°C for 8 minutes 20 seconds for a total of 16 cycles. The DpnI enzyme selectively digested the methylated parental plasmid, enriching the mutant plasmid.

Figure 5.3 :- SDM primers placed in the sequence

4.4. Transformation and Colony Screening

The DpnI-digested PCR product containing the mutated plasmid was transformed into E. coli DH5α. Eighteen colonies were obtained after overnight incubation. Replica plating was performed to preserve colonies for further analysis .

Figure 5.4 : Transformed Colony Growth on Ampicillin Plate

4.5. Confirmation of Mutation by Restriction Digestion

Plasmids were isolated from selected colonies and digested with EcoRI. Gel electrophoresis confirmed the presence of the expected three bands (3.6 kb, 2.8 kb, and 1.8 kb), indicating the presence of the complete plasmid with insert .

Figure 5.7:- Restriction Digestion of the mutated plasmid

4.6. Sequencing Confirmation

PCR was performed using mutation-specific primers, yielding a 310 bp product. The PCR product was purified and subjected to sequencing. Comparison of pre- and post-mutagenesis sequences confirmed the presence of the R248Q mutation (CGG → CAG) in the TP53 gene.

Figure 5.8:- PCR standardisation by the sequencing primers.

Figure 5.9:- Before Mutation Figure 5.10:- After Mutation

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Total Words: 383 **Total Characters:** 2587 **Plagiarized Sentences:** 0 **Unique Sentences:** 17 (100%)

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6. DISCUSSION

This study successfully generated a mammalian expression vector containing the R248Q mutation in the TP53 gene using site-directed mutagenesis. The mutation was introduced accurately and confirmed through sequencing, indicating that the mutagenesis and cloning steps were effective. This lays the groundwork for future studies aimed at exploring how this specific mutation alters p53 function in mammalian cells.

The R248Q mutation is one of the most frequently reported hotspot mutations in TP53 and is found in a wide range of human cancers. It affects the DNA-binding domain of p53, a region essential for its tumor-suppressing activity. By disrupting this domain, the R248Q variant is known to impair the ability of p53 to regulate target genes involved in controlling cell growth, inducing apoptosis, and maintaining genomic integrity. Constructing an expression vector that carries this mutation allows for its functional study in controlled cellular environments, helping to better understand how it contributes to cancer progression.

Introducing the mutant gene into a suitable vector makes it possible to express the altered p53 protein in mammalian cells. This opens up opportunities to examine its biological effects, such as changes in cell proliferation, resistance to cell death, and altered responses to DNA damage. Previous research has shown that the R248Q mutation can result in both a loss of tumor suppressor function and, in some cases, gain-of-function properties that promote tumor growth and invasion. Having a reliable model to study these effects will be valuable in identifying potential therapeutic targets.

While the vector construction was successful, there are a few limitations to acknowledge. This project focused on building and confirming the vector, but did not include functional assays to test the behavior of the mutant protein. Future work should involve transfecting the vector into mammalian cells and evaluating changes in gene expression, cell cycle dynamics, and other phenotypic effects. Additionally, although sequencing confirmed the presence of the R248Q mutation, a full plasmid sequence would be necessary to ensure no unintended changes occurred during cloning.

Overall, this project achieved its goal of constructing a TP53 R248Q expression vector, which will serve as a valuable tool for further research. It contributes to the growing body of work aimed at understanding how specific TP53 mutations influence cancer development and progression and provides a foundation for exploring targeted treatments in the future.

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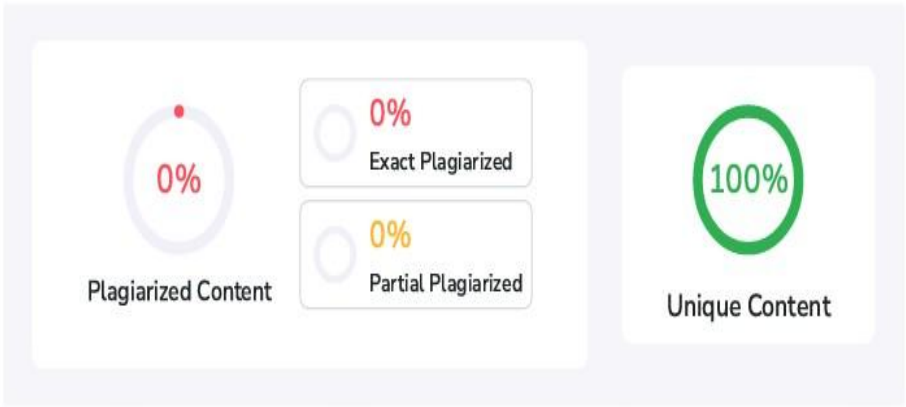

Total Words: 217 **Total Characters:** 1481 **Plagiarized Sentences:** 0 **Unique Sentences:** 9 (100%)

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7. CONCLUSION

This research successfully achieved the construction of a mammalian expression vector carrying the TP53 R248Q mutation through site-directed mutagenesis. The process was carefully carried out, and sequencing confirmed that the intended mutation was accurately introduced. This outcome marks an important step toward enabling more detailed studies on how this specific mutation affects the function of the p53 protein.

The R248Q mutation is widely known for its involvement in various types of cancer, primarily due to its location in the DNA-binding domain of p53—a region critical for its tumor-suppressive role. By creating a tool that allows controlled expression of this mutant protein in mammalian cells, this study helps pave the way for functional investigations that could deepen our understanding of p53-related cancer biology.

Although this project focused on the vector construction and verification, it lays the foundation for future work. The next logical step would be to study the biological effects of the R248Q mutation in cell-based models—looking at how it might influence cell growth, survival, or response to stress. These insights could eventually support the development of more targeted approaches for cancer treatment.

By providing a functional vector with a known cancer-associated TP53 mutation, it offers a valuable resource for future research aimed at exploring the role of mutant p53 and identifying potential therapeutic strategies